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# Integrin expression by primary and immortalized human chondrocytes: evidence of a differential role for α1β1 and α2β1 integrins in mediating chondrocyte adhesion to types II and VI collagen

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## Summary

*Objective:* Chondrocytes have been shown to express β1-containing integrins both *in vitro* and *in situ*, but their role in regulating chondrocyte function is poorly understood. The objective of this study was to determine how the relative expression of different integrins may be modulated in relation to the differentiated state and proliferative capacity of the chondrocyte.

*Design:* Integrin expression by four different cell lines of human chondrocytes immortalized with Simian virus 40 large T-antigen (SV40-TAg) was studied and compared to primary chondrocytes. Differences in α1 and α2 integrin subunit expression were utilized to further study the role of these integrins in mediating adhesion to types II and VI collagen.

*Results:* The overall cell-surface levels of β1-containing integrins were higher on all four immortalized cell lines which expressed over 10-fold higher levels of α2 and α3 integrin subunits compared to primary cells. However, primary cells expressed higher levels of the α1 integrin subunit which was not expressed by T/C28a4 cells and expressed at variable and lower levels in the other lines. Levels of the α3 integrin subunit were significantly greater on the highly proliferative juvenile costal chondrocyte lines (T/C-28a4, C-28I2, and C-20a4) compared to primary articular chondrocytes and tsT/AC-62 cells which were derived from adult articular chondrocytes. Expression of α5 was similar among primary cells and cell lines except on C-20/A4 cells which had an average of over 4-fold higher levels. None of the primary or immortalized chondrocytes tested expressed significant levels of α4. Cell adhesion assays revealed that both α1β1 and α2β1 could serve as chondrocyte adhesion receptors for types II and VI collagen. In cell lines expressing both integrins, α1β1 was the preferential receptor for type VI collagen while α2β1 was the preferential receptor for type II collagen. Rather than inhibiting adhesion, incubation with the α3 blocking antibody P1B5 increased adhesion of C-28/I2 cells to both fibronectin and type II collagen by 67% and 100% respectively.

*Conclusions:* Immortalization with SV40-TAg results in altered integrin expression by chondrocytes. Changes in the relative levels of α1, α2, and α3 subunits may significantly alter the manner in which chondrocytes interact with types II and VI collagen in the extracellular matrix.


*Key words*: Chondrocyte, Cartilage, Integrins, Collagen.

## Introduction

Chondrocytes are the only cell type present in cartilage and they are surrounded by an abundant extracellular matrix (ECM) which consists of several types of collagen (predominately types II, VI, IX, and XI), proteoglycans, and various glycoproteins including fibronectin. Through the selected synthesis and degradation of matrix components, chondrocytes are responsible for the maintenance of the cartilaginous matrix. It is becoming increasingly recognized that the extracellular matrix is an 'information rich' environment and interactions between the cell and ECM are important in regulating many biological processes which include cell growth, differentiation and survival.[1–3] The integrin family of cell surface adhesion receptors appears to play a major role in mediating cell-matrix interactions that are important in regulating these processes.

Integrins are heterodimeric transmembrane glycoproteins each consisting of one α and one β unit. The β1 subfamily of integrins contains many of the receptors for extracellular matrix proteins including receptors for fibronectin (α3β1, α4β1, α5β1), collagens (α1β1, α2β1, α3β1), and laminin (α3β1, α6β1).[4,5] Integrin binding stimulates intracellular signaling which can affect gene expression (outside-in signaling) and cells can alter the expression and affinity of their integrins (inside-out signaling). Various studies have documented expression of a number of different integrins by chondrocytes including α1β1, α2β1, α3β1, α5β1, α6β1, αVβ3, and αVβ5.[6–11] The precise role for these integrins in cartilage, however, is not completely understood.

Studies of human chondrocytes have been limited by the lack of availability of sufficient tissue, particularly normal adult cartilage, and by the instability in phenotypic expression of cultured chondrocytes. We sought to further study

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chondrocyte integrin expression and function using immortalized chondrocyte lines developed by stable transfection of primary human chondrocytes with Simian virus 40 large T-antigen (SV40-TAg) expression vectors. Immortalized chondrocytes have a high proliferative capacity which can be dissociated from their ability to express cartilage-specific matrix genes, such as type II collagen, under defined culture conditions.[12] Three lines (T/C-28a4, C-28/I2, and C-20/A4) have been developed using chondrocytes isolated from juvenile costal cartilage. These cell lines have been used to study a variety of chondrocyte functions.[13–17] A 4th line (tsT/AC-62) was developed recently using primary chondrocytes isolated from adult articular cartilage.[18] These latter cells were immortalized with a retrovirus expressing a temperature sensitive mutant of SV40-TAg which is functional when the cells are cultured at 32°C (permissive temperature) but not functional at 39°C (non-permissive temperature).

The T/C-28a4, C-28/I2 (a clonal line derived from the nonclonal T/C-28a4 cells) and C-20/A4 cells proliferate rapidly in medium containing 10% fetal bovine serum and under these conditions do not synthesize significant amounts of type II collagen. However, when the cells are switched to serum-free medium supplemented with an insulin-containing serum substitute, proliferation slows and production of type II collagen increases within 24–48 h without expression of type I collagen, a marker for 'de-differentiated' chondrocytes.[12,19] We analyzed expression of β1-containing integrins in these cell lines using flow cytometry and compared their patterns and levels of cell surface integrins to those on primary chondrocytes isolated from normal adult articular cartilage. All studies were performed with monolayer cultures that had just reached confluency. Differential expression of various integrin subunits was noted among the four cell lines and primary cells. The differential expression of α1 and α2 subunits was used to further explore the role of these integrins in mediating chondrocyte adhesion to types II and VI collagen.

## Materials and methods

### CELL CULTURE

Grossly normal appearing cartilage was obtained from knee joints within 6 h of death from five human tissue donors who did not have a known history of arthritis. Chondrocytes were isolated from the articular cartilage by digestion with pronase and collagenase as previously described.[20] The cells were cultured as high-density monolayers ($2\times10^5$ cells/cm$^2$) in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics. Cell culture medium and fetal bovine serum were from Gibco BRL (Gaithersburg, MD) and were purchased from the Tissue Culture Core Laboratory of the Wake Forest University Comprehensive Cancer Center. Except where indicated, cells were cultured to confluency (approximately 7 days). At this time point in culture integrin levels stabilize and the cells are still expressing a differentiated chondrocyte phenotype.[11]

The immortalized chondrocyte cell lines obtained by transfection of primary human chondrocytes with SV40-TAg have been described previously in detail.[12,18] Briefly, the C-20/A4 cells were immortalized by transfection with plasmid DNA expressing origin-defective SV40-TAg and derived by continuous passage from a single focus. The T/C-28a4 cells, from which the C-28/I2 clone was derived, were immortalized using a retrovirus expressing SV40-TAg and the neomycin-resistance selection marker (*neo*$^R$). These juvenile costal chondrocyte lines were maintained in a 5% $CO_2$ incubator at 37°C in DMEM supplemented with 10% FBS and were continuously passaged at approximately 90% confluency. Cultures allowed to grow to confluency were used for all experiments. In some experiments immortalized cells which had reached confluency were switched to serum-free DMEM supplemented with 1% Nutridoma (Boehringer Mannheim, Indianapolis, IN) and cultured for an additional 48 h.

tsT/AC-62 cells were developed using chondrocytes isolated from adult articular cartilage and were infected with a retrovirus expressing SV40-TAg and *neo*$^R$ (18, Robbins and Goldring, manuscript in preparation). The tsT/AC-62 cells express a temperature sensitive mutant of SV40-TAg which is functional when the cells are cultured at 32°C (permissive temperature) but not functional at 39°C (non-permissive temperature). These cells were cultured routinely at the permissive temperature of 32°C. For experiments examining the effect of functional SV40-TAg expression on integrin levels the near confluent cultures were switched to an incubator set at 39°C and the cells cultured for 18 days, a time point at which preliminary studies had noted increased type II collagen expression.

### GROWTH RATE ANALYSIS

Immortalized chondrocytes at approximately 80% confluence were trypsinized and replated in 6-well plates at 25,000 cells/well in 2-ml culture medium and cultured for up to 10 days at 37°C (T/C-28a4, C-28/I2, and C-20/A4) or 32°C (tsT/AC62). Triplicate wells containing cells from each line were trypsinized at 2 day intervals and the cells were counted using a hemocytometer.

### FLOW CYTOMETRY

Cells were harvested from confluent cultures with trypsin-EDTA, washed, counted and resuspended at $5\times10^6$ cells/ml in serum-free DMEM. One hundred μl of cell suspension was diluted 1:1 with PBS containing 0.2% BSA and 0.1% sodium azide. To this cell suspension, 5 μl of monoclonal anti-integrin antibody or an equivalent amount of mouse isotype control IgG (Sigma, St. Louis, MO) was added, and incubations were carried out for 45 minutes on ice. The integrin antibodies used were: anti-α1 (TS2/7 from T Cell Diagnostics, Woburn, MA); -α2 (P1E6), -α3 (P1B5), -α4 (P4G9) and -α5 (P1D6) from GibcoBRL; αV (MAB1980) from Chemicon (Temecula, CA) and anti-β1 (4B4) purchased from Coulter Corporation (Hialeah, FL). Cells were washed twice and then incubated for 30 minutes with FITC-conjugated anti-mouse IgG (Pierce, Rockford, IL). After rewashing and fixing in 2% paraformaldehyde, cells were analyzed on a FACScan cytofluorometer (Becton Dickinson, San Jose, CA). The relative fluorescence intensity was calculated from the log scale channel number for each sample. The specific fluorescence intensity was calculated by subtracting the relative fluorescence intensity of samples incubated with the control IgG from the relative fluorescence intensity obtained with specific integrin subunit antibodies. Results were analyzed using unpaired t-tests.

### REVERSE TRANSCRIPTASE PCR

The mRNA for α1 and α2 was detected using reverse transcriptase PCR as previously described in detail.[11] The

α1 primers framed a 391 bp sequence in the published human α1 cDNA starting at nucleotide 113 and were 5′ TCAATGTTGATGTGAAAAACT 3′ and 5′ CTATGTCCAGT TGAGTGCTGCATT 3′. The α2 primers framed a 420 bp sequence starting at nucleotide 2359 and were 5′ CTTGA AGCCTATTCTGAGACTGCC 3′ and 5′ GAAACTGAGAGA CGCCTGATTCTG 3′. Control primers for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were purchased from Clontech (Palo Alto, CA). Amplified sequences were analyzed on 1.6% agarose-ethidium bromide gels. For analysis of type II collagen and aggrecan mRNAs, total RNA was extracted using Trizol Reagent (GIBCO-BRL, Gaithersburg, MD) and reverse transcribed using Moloney Murine Leukemia Virus (MuLV) reverse transcriptase (Perkin Elmer, Foster City, CA) and oligo $d(T)_{16}$ as a primer in a final volume of 20 ul. For each set of primers, a PCR amplification was performed using 5 ul of the RT product in a final reaction volume of 50 ul containing 1 mM $MgCl_2$, 0.2 uM each of the upstream and downstream primers, and Taq DNA polymerase (Promega, Madison, WI). Following an initial denaturation at 95°C for 2 min, the DNA was amplified in the Perkin-Elmer GeneAmp PCR 9600 Thermocycler using 35 cycles of 30 sec at 95°C for denaturation and 1 min at 70°C for annealing, and a final extension at 72°C for 7 min, followed by cooling to 4°C. For GAPDH the annealing was performed for 30 sec at 60°C. Primer sets were as follows : (1) α1(II) collagen-5′-CCGAGGCAACGAT GGTCAGC-3′ and 5′-TGGGGCCTTGTTCACCTTTGA-3′ which gives a 359 bp product; (2) aggrecan-5′-TGAGG AGGGCTGGAACAAGTACC-3′ and 5′-GGAGGTGGTAAT TGCAGGGAACA-3′ which gives a 350 bp product[21] and (3) GAPDH-5′-GCTCTCCAGAACATCATCCCTGCC-3′ and 5′-CGTTGTCATACCAGGAAATGAGCTT-3′ which gives a 346 bp product.[21]

### CELL ATTACHMENT INHIBITION ASSAY

Cell attachment inhibition assays were performed using microtiter plates coated with 5 μg/ml fibronectin or 1 μg/ml of type II or VI collagen as described previously.[11,20] Primary human chondrocytes or immortalized chondrocytes were harvested from confluent cultures using low trypsin cell dissociation buffer (Gibco BRL) and washed with trypsin inhibitor in serum-free medium. After counting, the cells were diluted to $0.5\times10^6$ cells/ml in serum free media and incubated for 30 minutes with anti-integrin blocking antibodies at a concentration of 100 μg/ml or control IgG at the same concentration. The anti-integrin subunit blocking antibodies used were: anti-α1 (5E8D9) from Upstate Biologicals Inc. (Lake Placid, NY); -α2 (MAB 1998) from Chemicon; -α3 (P1B5) and -α5 (P1D6) from GibcoBRL. The cell suspension (100 μl) was transferred to coated wells, allowed to incubate for 45 min at 37°C, and then adherent cells were quantified using an assay for hexosaminidase as described.[20] Attachment assays were performed in duplicate wells and the results are provided as the mean and s.e.m. of at least three independent experiments performed with each antibody.

### STATISTICAL ANALYSIS

Results from the flow cytometry experiments and adhesion assays were analyzed by analysis of variance (ANOVA) using StatView software (SAS Institute, Cary, NC). Significance was set at 0.05 and Fisher PLSD was used as the post hoc test.

## Results

### PROLIFERATIVE CAPACITY AND CARTILAGE-SPECIFIC MATRIX GENE EXPRESSION IN IMMORTALIZED CHONDROCYTE LINES

The morphology of the four immortalized chondrocyte lines is shown in Fig. 1. The T/C-28a4 cells (Fig. 1A) are the most rounded of the four lines and therefore the most similar morphologically to primary chondrocytes while the tsT/AC 62 cells (Fig. 1D) are the most flattened and spread with a more fibroblastic appearance. As shown in Fig. 2A, the T/C-28a4, C-28/I2, and C-20/A4 lines are highly proliferative when cultured in serum-containing medium while the tsT/AC-62 cells have a relatively low proliferative rate, even when cultured at the permissive temperature (32°C) for functional SV 40 expression. Similar to primary chondrocytes, all four lines are capable of expressing cartilage matrix-specific genes including type II collagen and aggrecan (Fig. 2B). Levels of mRNA for type II collagen significantly increased in the C-28/I2 cells when they were switched from culture in medium containing 10% serum to serum-free medium supplemented with 1% Nutridoma. A modest increase in type II expression was also noted when the T/C-28a4 cells were switched to 1% Nutridoma.

### EXPRESSION OF β1-INTEGRINS BY IMMORTALIZED CHONDROCYTES

The cell surface expression of β1 and α1–α5 integrin subunits was analyzed by flow cytometry using the four SV40-TAg immortalized chondrocyte cell lines cultured to confluence in monolayer. Levels of integrins on the immortalized chondrocytes were compared to those on confluent cultures of primary articular chondrocytes isolated from normal human articular cartilage. As demonstrated in Fig. 3 and summarized in Table I, several significant differences in the levels of specific integrin subunits were noted both between immortalized and primary chondrocytes and among the various immortalized lines.

Levels of the α1 integrin subunit were significantly higher on primary chondrocytes than on T/C-28a4 cells ($P<0.0001$), C-28/I2 cells ($P<0.05$), C-20/A4 cells ($P<0.002$), and the tsT/AC-62 cells at 32°C ($P=0.02$) but not the tsT/AC-62 cells after culture for 18 days at 39°C. The latter conditions are nonpermissive for functional SV40-TAg expression and cell proliferation.[18] The T/C-28a4 cells differed from the other lines in that they did not express any α1 integrin subunit. No mRNA for α1 could be detected in these cells by RT-PCR (Fig. 4A). Levels of α1 measured by flow cytometry did not change when T/C-28a4 cells or C-28/I2 cells were cultured for an additional 48 h in serum-free medium supplemented with 1% Nutridoma.

Consistent with previous reports,[6,9,11] primary adult articular chondrocytes did not express measurable cell surface levels of the α2 integrin subunit. RT-PCR using RNA obtained from freshly isolated primary human chondrocytes showed weak to no signal for α2 mRNA even after 40 cycles of amplification (Fig. 4B). In contrast, all of the immortalized chondrocyte lines expressed significant cell surface levels of α2 including the tsT/AC-62 cells which were developed from cells isolated from adult articular cartilage, unlike the other lines which were developed from cells isolated from juvenile costal cartilage. Culturing the tsT/AC-62 cells at the non-permissive temperature did not significantly reduce levels of α2. There was a modest reduction in α2 levels when the T/C-28a4 and C-28/I2 cells

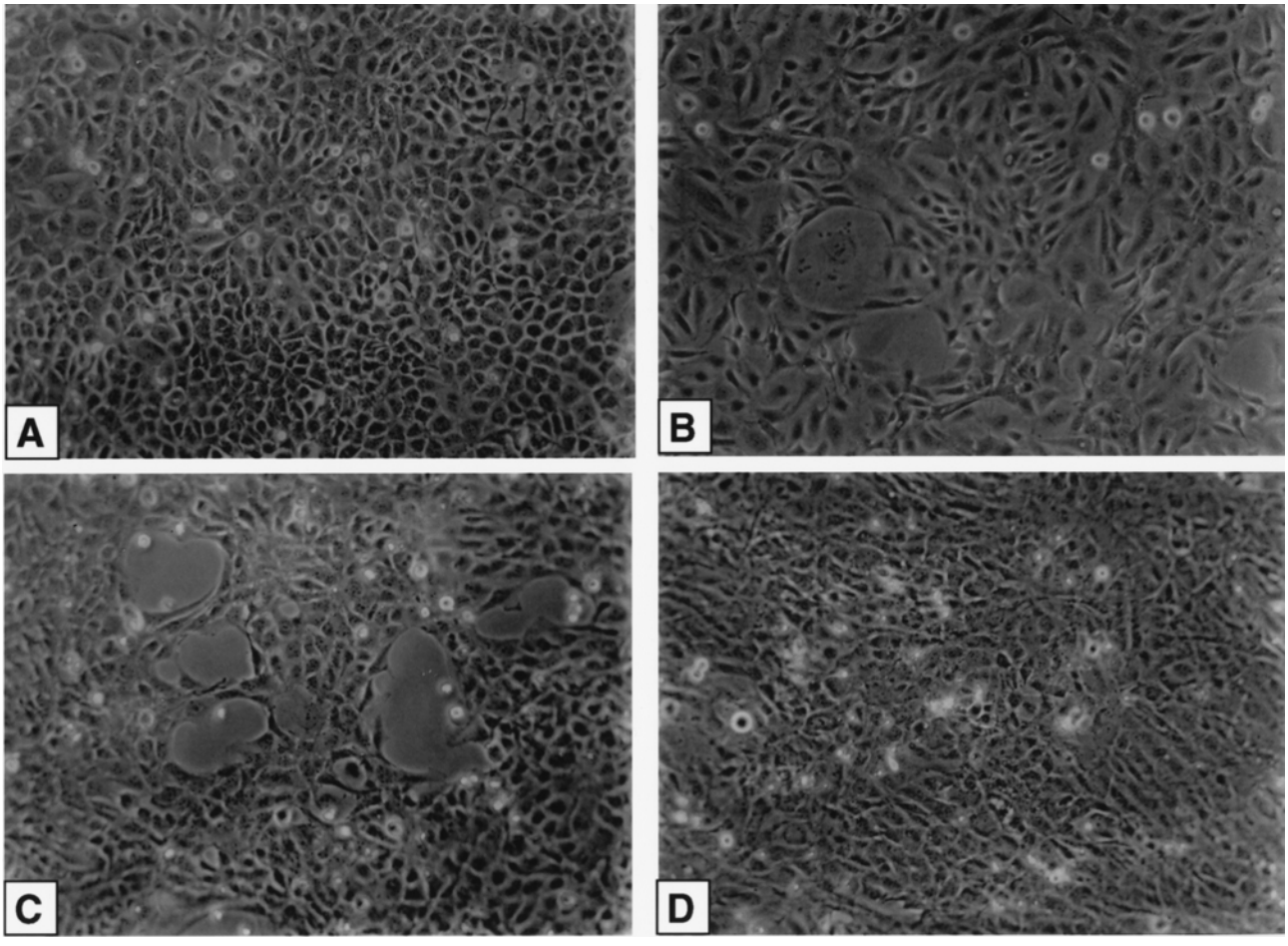


Fig. 1. Morphology of immortalized chondrocyte cultures. (A) T/C-28a4, (B) C-28/I2, (C) C-20A4, and (D) T/AC62 cells were cultured to confluency and photographed using phase-contrast microscopy. The T/C-28a4 cells are the most rounded while the T/AC62 cells are very flattened and appear more fibroblastic.

were cultured in serum-free medium supplemented with Nutridoma, however differences were not statistically significant.

A dramatic difference between primary and immortalized chondrocytes was seen when α3 integrin subunit expression was analyzed. Levels of this integrin subunit were significantly higher on all of the immortalized chondrocyte lines compared to primary cells ranging from a greater than 5-fold increase in the tsT/AC-62 cells ($P=0.05$) to a 16-fold increase in the C-20/A4 cells ($P<0.0001$). Similar to results with α2, prolonged culture of the tsT/AC-62 cells at the non-permissive temperature did not reduce the levels of α3 while there was a modest reduction when T/C-28a4 and C-28/I2 cells were cultured in serum-free medium supplemented with Nutridoma which was statistically significant for the C-28/I2 cells ($P=0.02$).

Unlike α1–α3, the levels of the α5 integrin subunit were similar between primary and immortalized chondrocytes except for the C-20/A4 cells which expressed over 4-fold higher levels of α5 ($P<0.0001$). Finally, the levels of the β1 integrin subunit were significantly higher on all the immortalized chondrocyte lines compared to primary cells ($P<0.006$) and were highest on the tsT/AC-62 cells with a further increase after prolonged monolayer culture at the non-permissive temperature ($P=0.006$, tsT/AC-62 cells at 32° vs 39°C). Under these latter conditions the tsT/AC-62 cells were very flattened and difficult to remove from the culture dish requiring a prolonged incubation with trypsin-EDTA.

Expression of αV was measured on primary cells and the T/C-28a4, C-28/I2, and C-20/A4 lines and found to be present at similar levels except for C-28/I2 cells which had higher levels than the other two lines ($P<0.02$) (data not shown). The possibility that contaminating fibroblasts may be accounting for the altered integrin expression profile in the immortalized cells was assessed by measuring levels of the α4 integrin subunit which is expressed at significant levels by synovial fibroblasts.[22] Neither the C-20/A4 or tsT/AC-62 immortalized lines or primary chondrocyte cultures expressed α4 by flow cytometry (Table I).

### EFFECTS OF INTEGRIN BLOCKING ANTIBODIES ON CHONDROCYTE ADHESION

The finding of differential expression of integrins among the four immortalized lines and primary chondrocytes was used to assess the role of the α1- and α2-containing integrins in mediating chondrocyte adhesion to types II and VI collagen. Cells were incubated with antibodies known to block either the α1 or α2 integrin subunit and adhesion to type II or VI collagen was determined in a standard cell adhesion assay. Adhesion-blocking results correlated well with the results of flow cytometry in that adhesion of cells

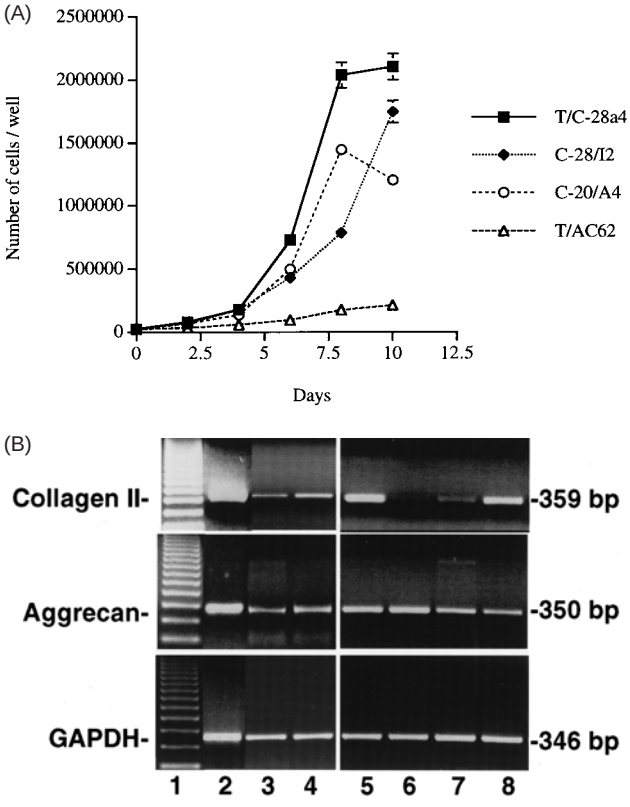


Fig. 2. Proliferative capacity and matrix gene expression in immortalized chondrocyte lines. (A) The four immortalized chondrocyte lines were plated in DMEM with 10% FBS at 25,000 cells/well in 6-well plates. At the indicated time points cells were harvested from the wells using trypsin-EDTA and counted using a hemocytometer. Results shown are the means and s.e.m. of triplicate determinations. (B) Total RNA was isolated from immortalized chondrocyte lines and used for RT-PCR to detect expression of type II collagen and aggrecan. Expression of GAPDH was used as control. Lane 1: DNA standards; lane 2: primary adult articular chondrocytes; lane 3: T/C-28a4 cells cultured in medium with 10% FBS; lane 4: T/C-28a4 cells in serum-free medium supplemented with 1% Nutridoma; lane 5: C-28/I2 cells in 1% Nutridoma; lane 6: C-28/I2 cells in 10% FBS; lane 7: C-20A4 cells in 10% FBS; lane 8: T/AC62 cells in 10% FBS at 32°C.

which did not express a particular integrin subunit was not blocked by antibodies to that subunit. Significant inhibition of adhesion to type II collagen ranging from 62–79% was noted when the immortalized cells were incubated with the α2 antibody (Table II). In contrast, the α1 integrin subunit blocking antibody was less effective in blocking adhesion to type II collagen. No inhibition of adhesion of the T/C-28a4 cells or C-20/A4 cells to type II collagen was noted with the α1 antibody, and only 26% inhibition of adhesion of the C-28/I2 cells was noted with anti-α1 (Table II). Consistent with previous reports from our lab,[11] a mean of 38% inhibition to type II collagen was observed when primary chondrocytes were incubated with the α1 antibody and no inhibition was noted using the α2 antibody. The difference between the α1 and α2 antibodies in blocking adhesion to type II collagen was significant at $P<0.0001$ for all cell lines. When the α1 and α2 antibodies were combined, the inhibitory effects were additive in cells expressing both integrin subunits resulting in 93% inhibition of adhesion of the C-28/I2 cells and 87% inhibition for C-20/A4 cells.

The relative effects of the α1 and α2 antibodies were different when the adhesion blocking assays were performed using type VI collagen instead of type II collagen. The α1 antibody was more effective in blocking adhesion to type VI collagen than the α2 antibody in cells expressing both integrins (Table III). The difference between the two antibodies was significant for all cell lines at $P<0.01$. As expected the α1 antibody did not block adhesion of the T/C-28a4 cells which do not express α1 but did block 62–74% of the adhesion using the cells which did express α1. The α2 blocking antibody was most effective against adhesion of the T/C-28a4 cells (79% inhibition) and as expected did not significantly inhibit adhesion of primary chondrocytes which do not express α2. Similar to the findings with type II collagen, use of both α1 and α2 antibodies together resulted in additive effects in cells expressing both integrins.

The potential differences between α1 and α2 in mediating adhesion to types II and VI collagen were further explored by directly comparing primary chondrocytes (α1+ and α2−) to T/C-28a4 cells (α1− and α2+). In adhesion assays using collagen concentrations from 0.1 to 10 ug/ml, more T/C-28a4 cells attached to type II collagen relative to primary cells while more primary cells attached to type VI collagen (Fig. 5A, B). Likewise, when adhesion to type VI collagen was studied using a range of cell concentrations, more primary cells adhered to type VI collagen compared to T/C-28a4 cells (Fig. 5C).

Given the high level of expression of the α3 integrin subunit by the cell lines, it was of interest to determine if this integrin subunit was serving as an alternative type II collagen or fibronectin receptor as has been noted with other cell types.[23,24] As shown in Fig. 6, incubation of C-28/I2 cells with a blocking antibody to the α3 integrin subunit significantly increased adhesion to both fibronectin (mean 167% of control, $P=0.001$) and type II collagen (mean 204% of control, $P<0.0001$). Incubation of the cells with an α5 blocking antibody inhibited adhesion to fibronectin by about 50% ($P=0.01$) but did not affect adhesion to type II collagen. The combination of α3 and α5 gave similar results to α3 alone.

## Discussion

Immortalization with SV40-TAg stabilizes the proliferative phenotype of cells and, as shown here, results in an alteration in integrin expression. Although all of the immortalized chondrocyte cell lines were produced by stable expression of SV40-TAg, differences in levels of specific integrin subunits among the four lines were found. When compared to primary chondrocytes isolated from normal adult articular cartilage, the immortalized lines exhibited an overall increase in integrin expression particularly in the expression of the α2 and α3 subunits. However, levels of the α1 integrin subunit were highest on the primary chondrocytes and varied greatly among the four immortalized chondrocyte lines. As will be discussed further, these differences in integrin expression could either reflect differences in the sources of the cells or the effects of immortalization.

Chondrocyte expression of α1β1 and α2β1 integrins has varied among published studies depending on the source of the cells. Adult human articular chondrocytes were shown to express α1β1 but not α2β1[6,9] while human fetal articular,[8,25] human chondrosarcoma,[10,26] and bovine calf[10] chondrocytes expressed α2β1 and little or no α1β1.

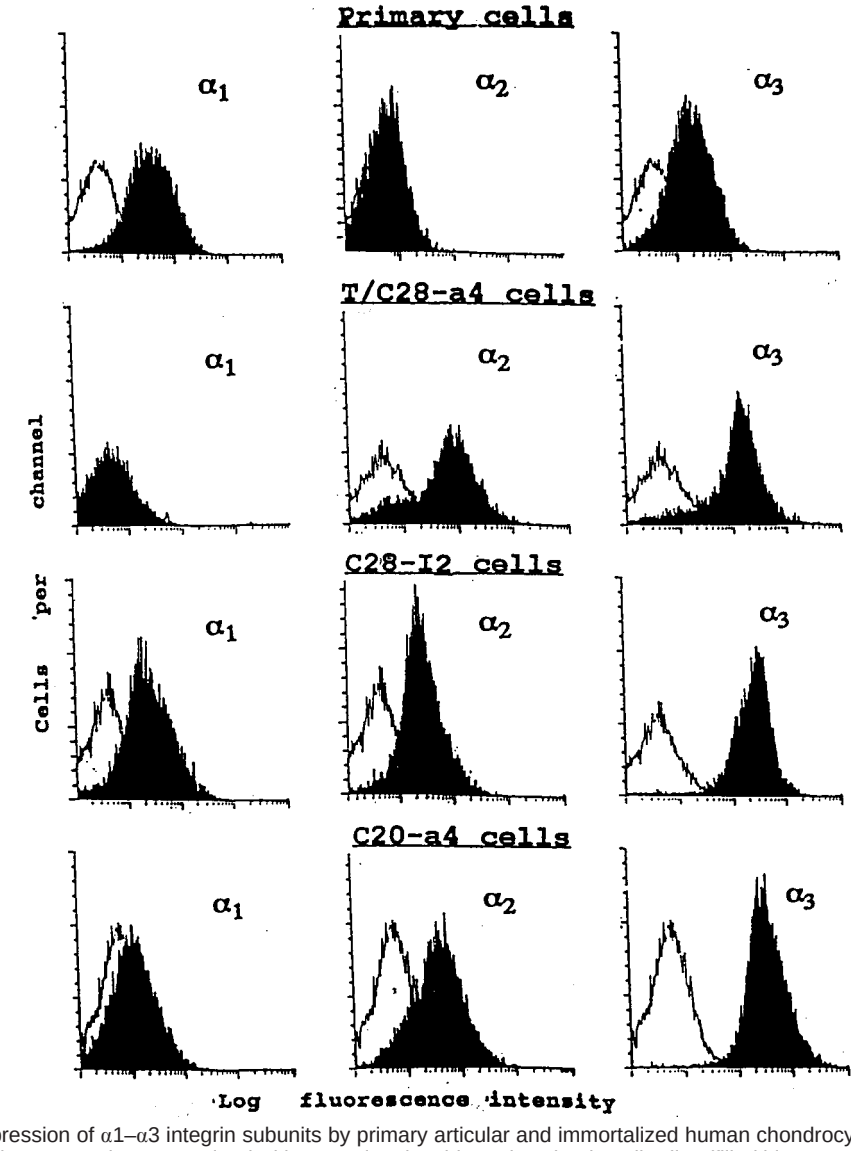


Fig. 3. Cell surface expression of α1–α3 integrin subunits by primary articular and immortalized human chondrocytes. Cells harvested from confluent monolayer cultures were immunostained with monoclonal anti-integrin subunit antibodies (filled histograms) or with isotype-specific control mouse IgG (open histograms) followed by FITC-conjugated secondary antibody and flow cytometric analysis.

Table I
*Analysis of chondrocyte integrin levels by flow cytometry**

| Cell line | α1 | α2 | α3 | α4 | α5 | β1 |
|---|---|---|---|---|---|---|
| Primary | 32 ± 4 | 3± 1 | 16± 0.3 | 2±0.7 | 19± 5 | 17± 0.5 |
| T/C-28a4 | 0.4± 0.1 | 47±13 | 141±12 | NT | 18± 3 | 56± 7 |
| Serum-free | 0.5± 0.2 | 31± 1 | 93±13 | | 15± 3 | 44± 6 |
| C-28/I2 | 20 ± 1 | 21± 2 | 206±22 | NT | 14± 3 | 94± 8 |
| Serum-free | 21 ± 2 | 15± 3 | 130±22 | | 10± 2 | 64±15 |
| C-20/A4 | 9 ± 2 | 28± 2 | 260±41 | 5±0.6 | 83±11 | 66± 2 |
| T/AC-62 32° | 14 ± 2 | 48± 8 | 85±12 | 9±1.3 | 28± 5 | 162±37 |
| 18 days 39° | 28 ±16 | 32±19 | 89±11 | NT | 23±11 | 226±18 |

*Results are mean±s.e.m. of specific relative fluorescence intensity ($n$=3 independent experiments, except for T/AC-62 cells where $n$=2). Except where indicated as serum-free, cells were cultured to confluence in DMEM with 10% FBS. Serum-free cultures were incubated for 48 h in DMEM supplemented with 1% Nutridoma. NT=not tested.

Our results showing expression of α1 and no α2 integrin in cultures of primary adult articular chondrocytes, are consistent with those of Woods *et al*.[9] and Salter *et al*.[6] but differ from Yonezawa *et al*.[27] who reported low levels of both α1 and α2 by flow cytometry on adult articular chondrocytes. Since the T/C-28a4, C-20/A4, and C-28/I2

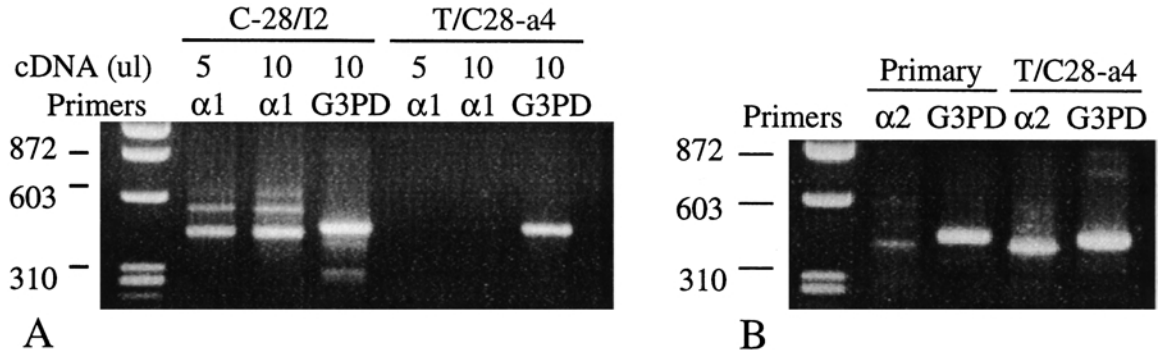


Fig. 4. RT-PCR analysis of α1 and α2 integrin subunit expression. Total RNA (0.5 μg per sample) isolated from cultured cells was reverse transcribed to cDNA which was amplified using primers specific for the α1 integrin subunit (A) or the α2 integrin subunit (B) with G3PDH primers as a control. The PCR products were separated on a 1.6% agarose-ethidium bromide gel along with φ X 174/Hae III standards shown in the far left lanes. (A) C-28/I2 cells, which were α1 positive by flow cytometry were compared to the α1 negative T/C-28a4 cells. (B) Primary adult articular chondrocytes, which were α2 negative by flow cytometry, were compared to the α2 positive T/C-28a4 cells. In (A) two amounts (5 ul and 10 ul) from the reverse transcription reaction were amplified by PCR while in (B) a single tube reaction system was used.

Table II
*Effects of α1 and α2 integrin blocking antibodies on adhesion of chondrocyte lines to Type II collagen**

| Cell line | Antibody | | |
|---|---|---|---|
| | α1 | α2 | α1+α2 |
| T/C 28a4 | 107± 6 | 28±6 | 36±12 |
| C28/I2 | 74±12 | 21±7 | 7± 3 |
| C20/A4 | 146±20 | 38±8 | 13± 6 |
| Primary | 62± 3 | 116±2 | NT |

*Results are the % adhesion (mean±s.e.m.) of cells incubated with the specified integrin subunit antibodies relative to control cells incubated with equivalent amounts of non-specific IgG. At least 3 independent experiments were performed with each cell line and antibody.

Table III
*Effects of α1 and α2 integrin blocking antibodies on adhesion of chondrocyte lines to Type VI collagen**

| Cell line | Antibody | | |
|---|---|---|---|
| | α1 | α2 | α1+α2 |
| T/C 28a4 | 94±10 | 21± 6 | 23±12 |
| C28/I2 | 38± 3 | 65± 7 | 18± 3 |
| C20/A4 | 34± 5 | 69± 6 | 1± 1 |
| Primary | 26± 8 | 93±11 | NT |

*Results are the % adhesion (mean±s.e.m.) of cells incubated with the specified integrin subunit antibodies relative to control cells incubated with equivalent amounts of non-specific IgG. At least 3 independent experiments were performed with each cell line and antibody.

lines were all produced from juvenile costal chondrocytes rather than adult articular cartilage, the relative levels of α1 and α2 found on these cells could reflect the tissue source. But the tsT/AC-62 cells, which also expressed significant levels of α2, were derived from cells isolated from adult articular cartilage. Therefore, α2 expression by these cells more likely is due to effects of immortalization and selection of proliferative cells. Expression of α2 by the immortalized cells is consistent with findings in chondrosarcoma cells, which are transformed, and with fetal chondrocytes and chondrocytes from young calves which all have a greater proliferative capacity than adult articular chondrocytes.

Santala *et al*.[28] reported induction of α2β1 in both virally and chemically transformed human osteosarcoma cells and suggested that a negative regulator of α2 expression was lost after transformation. It seems likely that the same or a similar negative regulator is lost when chondrocytes are immortalized. Expression of α2 in the immortalized chondrocyte lines is not due directly to the effects of SV40-TAg expression, since α2 levels were maintained in the tsT/AC-62 cells even after prolonged culture at the nonpermissive temperature for functional SV40-TAg expression. Maintenance of α2 expression under these conditions and the finding that α2 expression was found in cells expressing type II collagen and aggrecan argues against this integrin subunit being a marker of 'dedifferentiated' chondrocytes. A recent study be Ostergaard *et al*.[29] reported the presence of α2 in human osteoarthritic cartilage. We have been unable to detect α2 by flow cytometry on chondrocytes isolated from osteoarthritic cartilage or on adult articular chondrocytes passaged up to six times in monolayer culture, conditions known to cause an alteration in expression of the differentiated phenotype (unpublished observations). Since we did detect low levels of α2 mRNA in primary adult articular chondrocytes it is possible, however, that primary cells could express significant cell surface levels of α2 under conditions which we have not tested. Since fetal chondrocytes express α2 and since α2 has been noted on primary adult chondrocytes by other investigators during conditions different from ours, the finding that the immortalized lines express α2 does not imply that these cells are not suitable for understanding chondrocyte function. Rather, the results further demonstrate that integrin expression by chondrocytes can be altered by many factors which include not only immortalization and differentiation but also cell culture,[10,11,35] growth factors,[30] and in osteoarthritic cartilage.[11,29] The results, however, indicate that studies using the immortalized chondrocytes, particularly when cell-ECM interactions are involved, need to consider the potential affects of the differences in integrin expression.

The differential expression of α1 and α2 integrin subunits was utilized in the present study to further determine the role of these integrins in mediating chondrocyte adhesion to types II and VI collagen. Both α1β1 and α2β1 integrins are well recognized collagen receptors in other cell types, and both have been shown to mediate chondrocyte adhesion to type II collagen.[8,10,11,26] The α1β1 integrin has

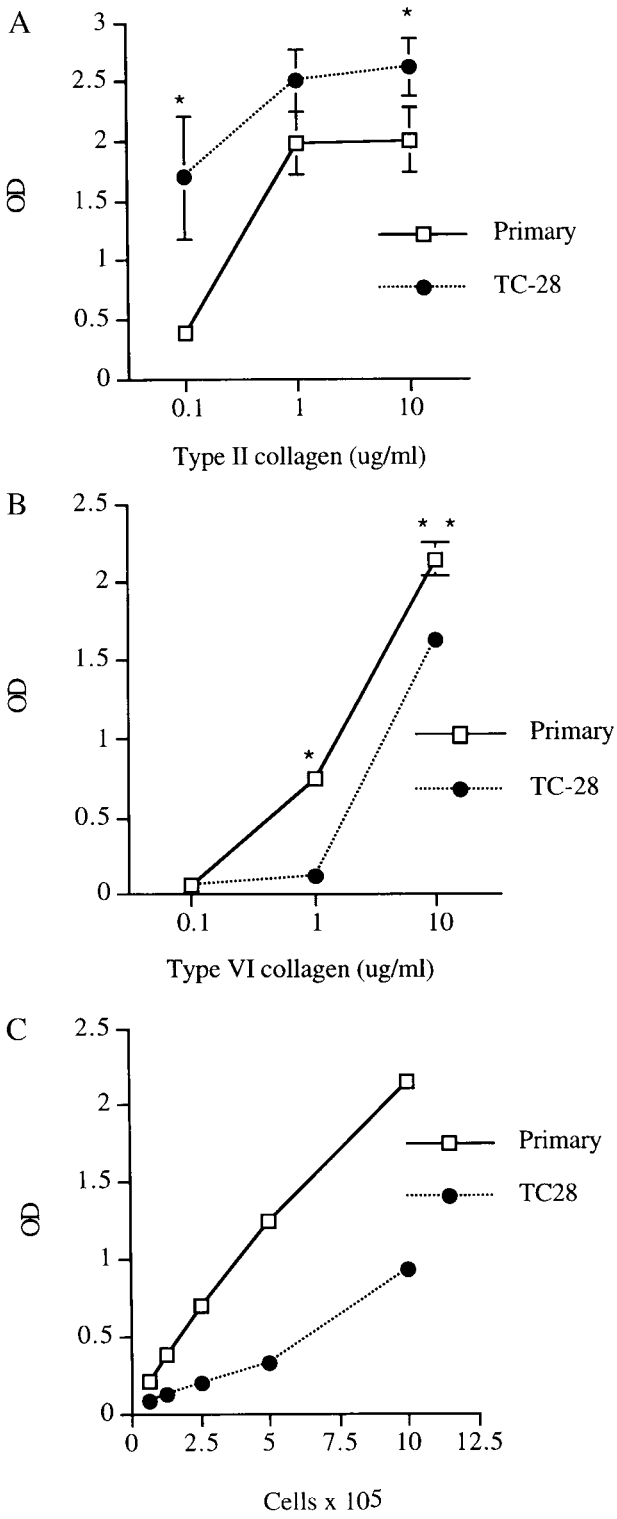


Fig. 5. Adhesion of primary human articular chondrocytes and immortalized T/C-28a4 cells to types II and VI collagen. (A) and (B) Primary chondrocytes and T/C-28a4 cells at $1\times10^6$ cells/ml were added to microtiter wells coated with type II collagen (A) or type VI collagen (B) at the indicated concentrations. Results are shown as OD units (mean±s.e.m.) from the hexosaminidase assay used to quantitate adherent cells. (C) Primary chondrocytes and T/C-28a4 cells at the indicated concentrations were added to wells coated with 10 ug/ml type VI collagen and adhesion was quantitated using the hexosaminidase assay (*$P$<0.002, **$P$=0.02 for differences between primary and T/C-28a4 cells).

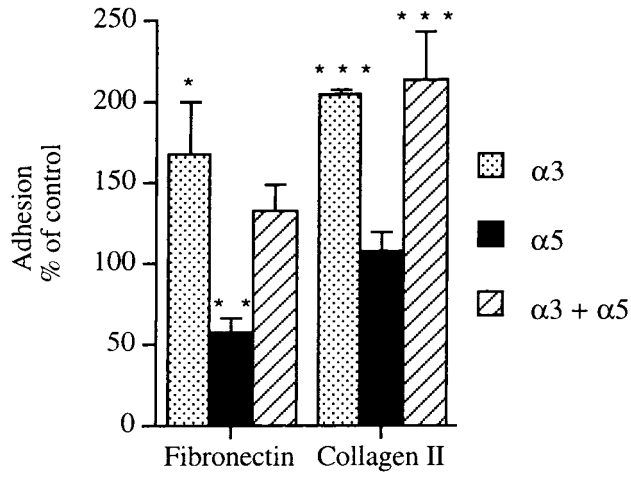


Fig. 6. Effects of anti-α3 and -α5 integrin blocking antibodies on C-28/I2 adhesion to fibronectin and type II collagen. The C-28/I2 immortalized chondrocytes were harvested from culture and incubated with 100 ug/ml of monoclonal anti-α3 and/or -α5 integrin blocking antibodies or nonspecific mouse IgG as control. Cells and antibodies were then incubated in wells coated with fibronectin or type II collagen for 45 minutes and adherent cells were measured using the hexosaminidase assay. Results are the means and s.e.m. of three independent experiments expressed as a % of control adhesion (*$P$=0.001, **$P$=0.01, ***$P$<0.0001 for differences between integrin antibody treated and control).

also been shown to mediate binding of primary adult articular chondrocytes to type VI collagen.[30] The cell adhesion blocking assays using primary chondrocytes and three of the immortalized lines confirmed that both integrins can mediate adhesion to both types of collagen. In addition, these studies revealed that the α1 integrin subunit was responsible for a greater percentage of adhesion to type VI collagen in cells expressing both α1 and α2, while the opposite was true for type II collagen where α2 mediated a greater percentage of adhesion to type II collagen. The T/C-28a4 cells which expressed α2 and no α1 and primary chondrocytes which expressed α1 and no α2 served as excellent controls for these studies. Adhesion assays comparing these cells revealed differences in numbers of cells adhering to types II and VI collagen further substantiating an important role for α1β1 in mediating adhesion to type VI collagen and α2β1 in adhesion to type II collagen.

This is the first report showing differences in preferential binding of α1 and α2 to types II and VI collagen. A recent study[31] using transfected CHO cells demonstrated that differences in the I-domains in α1 and α2 result in differential affinity for types I and IV collagen with α1 showing preference for type IV (a non-fibrillar collagen) and α2 a preference for type I (a fibrillar collagen) similar to our results showing a preference of α1 for type VI (a non-fibrillar collagen) and α2 for type II (a fibrillar collagen). These results suggest that changes in the expression of α1β1 and α2β1 integrins may be an important mechanism by which cells can alter their interactions with types II and VI collagen in cartilage. Such a switch in integrin expression and collagen binding may be occurring as fetal chondrocytes mature into adult chondrocytes.

Since only 38% adhesion inhibition to type II collagen was noted with primary adult chondrocytes using the α1 blocking antibody and since these cells did not express α2, additional integrin receptors for type II collagen on these cells must exist. It is unlikely that this represents adhesion

mediated by anchorin CII (annexin V), since annexin V does not appear to bind pepsin-treated collagen,[32] which was the form of collagen used in our adhesion assays and also in the studies by Durr *et al.*,[8] Holmvall *et al.*,[10] and Tuckwell *et al.*[26] which all suggested the presence of additional type II collagen-binding integrins. In previous work we reported >90% type II collagen adhesion inhibition when primary chondrocytes were incubated with a β1 integrin blocking antibody, suggesting that type II collagen binding is mediated by an additional α subunit which associates with β1.[20] Recently the discovery of a new integrin, α10β1, was reported.[33] This integrin, along with α1β1, was purified from bovine chondrocytes using a type II collagen affinity column suggesting that it also mediates chondrocyte adhesion to type II collagen. Blocking antibodies are not yet available to α10 but it is likely that this integrin subunit could be an important mediator of type II collagen binding by primary adult articular chondrocytes.

Expression of the α5β1 integrin by chondrocytes has been a consistent finding and it appears to be a primary chondrocyte receptor for fibronectin. Enomoto-Iwamoto *et al.*[34] suggested that α5β1 might be involved in regulating chondrocyte proliferation since colony formation by rabbit growth plate chondrocytes in soft agar was inhibited by RGD peptides and by an α5β1 blocking antibody which inhibit fibronectin adhesion. If α5β1 does play a role in regulating chondrocyte proliferation, our results would indicate that proliferation is unlikely to be controlled simply by the level of α5β1 on the cell surface, since similar levels of this integrin were found on primary chondrocytes and the highly proliferative immortalized lines.

It is possible, however, that cell surface levels of the α3 integrin subunit play a role in regulating chondrocyte proliferation and matrix deposition. The levels of this integrin were much higher on the immortalized chondrocyte lines, with a 16-fold difference noted between primary chondrocytes and the C-20/A4 cells. Of the four immortalized lines tested, C-20/A4 cells deposit the least amount of extracellular matrix (12 and unpublished observations). Also, levels of α3 decreased when T/C-28a4 and C-28/I2 cells were changed to serum-free medium supplemented with Nutridoma which reduces the rate of proliferation and increases matrix synthesis in these cells. The tsT/AC-62 cells, which are the least proliferative of the four lines studied, also had the lowest levels of α3. The lack of change in α3 levels when these cells were switched to the non-permissive temperature could argue against a correlation of α3 levels with proliferation and matrix deposition. However, these cells proliferate much more slowly than the other immortalized lines even at the permissive temperature. Tavella *et al.*,[35] reported that α3 levels were high in dedifferentiated chick chondrocytes and decreased as the cells differentiated, conditions also associated with a reduction in proliferation and an increase in matrix synthesis. It is interesting that in the adhesion inhibition studies, blocking α3 actually increased adhesion of C-28/I2 cells to both fibronectin and type II collagen by 67% and 100% respectively. In similar studies using a breast carcinoma cell line it was found that blocking α3 increased collagen but not fibronectin adhesion.[36] The authors of the latter study suggested the possibility of negative cooperativity between α2β1 and α3β1 integrins. Together, these results suggest that α3 may regulate proliferation by reducing adhesive interactions between chondrocytes and their extracellular matrix resulting in increased proliferation and reduced matrix deposition.

In summary, immortalization of human chondrocytes with SV40-TAg results in cell surface levels of β1-containing integrins which differ from primary adult articular chondrocytes. The differential integrin expression noted in the four immortalized lines tested proved advantageous in studies assessing the roles of α1β1 and α2β1 integrins in mediating adhesion to types II and VI collagen. The regulated expression of these integrins during chondrogenesis may play an important role in how the cells interact with their extracellular matrix. Integrin-mediated cell-matrix interactions appear to play a key role in both chondrocyte survival and differentiation as demonstrated by recent studies in which chick sternal cartilage in organ culture was treated with blocking antibodies to the β1, α2, and α3 integrin subunits.[37] Future studies defining the cell signaling pathways activated by stimulation of specific integrins should provide increased understanding of how the various integrins expressed by chondrocytes regulate chondrocyte growth, differentiation, and survival.

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